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Dental Technology Dissertation Project

BSc(Hons) in Dental Technology

In-Vitro Cytotoxicity Evaluation of Dental Ceramics' Surface Characterisation

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A report submitted in the part fulfilment for the degree of Bachelor of Science (Hons) in Dental Technology

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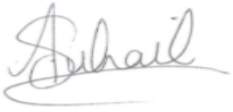

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Abstract

Introduction

The use of dental glaze and shades on all-ceramic materials is crucial to achieve sufficient levels of aesthetics for restorations. However, their biocompatibility and impact on the oral environment remains a topic of limited research. The purpose of this experiment is to explore any potential cytotoxicity caused by the use of extrinsic staining on dental ceramics, using an *In-Vitro* methodology.

Materials and Methods

Surface characterisation materials for glass-ceramics and zirconia were assessed. A variety of 8 group combinations of glaze and stains were applied onto glass-ceramic specimens (Celtra, n=7, e.max, n=4) and Zirconia specimens (Katana, n=7). Following the fabrication, the treated specimens were allowed to leach potential ions into serum-free media for 72 hours. LDH assay was used to test the cytotoxicity produced using human gingival fibroblast or human dermal fibroblasts.

Results

Based on the results from the pilot study using the e.max materials, a positive correlation was found between the cytotoxicity and the application of extrinsic glazing and staining procedures. The *p* value of 0.005 was lower than the threshold we set at 0.05, which provided evidence for the cytotoxicity. The results for the Katana and Celtra were disregarded due to complications during the methodology.

Conclusion

It can be concluded that cytotoxicity to cells was caused by the application of external glaze and shades. This was due to an increased amount of LDH released following contact with the media containing leached ions from the surface of the treated specimens.

However, further research must be conducted to gain greater knowledge on this subject area.

Acknowledgments

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1.0 Introduction

1.1 Fixed Prosthodontics

Restorative dentistry has the purpose of re-establishing the aesthetics and function of the oral cavity, by the means of utilising different dental appliances and materials. Dental devices range from all-ceramic crown and bridge work, to cobalt-chromium removable prosthetics. The extent of treatment needed, and the requirements of the patient are determining factor in the selection of appliances. Properties of materials such as biocompatibility, longevity, chemical reactivity, mechanical stability and durability must be thoroughly assessed to prevent damage to the gingivae and surrounding tissue. Surface treatments used for external characterisations; such as stains and glazes are essential in producing an aesthetic restoration. These can be applied internally or externally on the surface of the dental work. However, the limited amount of research and testing present on these materials may raise concerns regarding their suitability for use in the oral environment, and their ability to cause reactions with soft oral tissue (Gociu et al, 2013.,).

1.2 Metal-Ceramic Restorations

Metal-Ceramic crowns have been a highly reliable and popular choice for dental clinicians and technicians for many decades, as they show large quantities of triumph and success with patients. This may therefore lead to the argument that the transition to all-ceramics has been rushed (Christensen, 2014.,). Although their strength has been praised, they also present many disadvantages, which questions their suitability to use for fixed prosthetic restorations. The presence of non-precious alloys consisting of constituents including nickel can cause hypersensitivity for some patients and can exhibit corrosive properties. Furthermore, the aesthetics also present an issue due to the visibility

of the underling metal framework through the translucent layering ceramic. This reduces the suitability of the material for anterior restorations (Canadian Agency for Drugs and Technologies in Health, 2015.,). However, the recent development and research into all-ceramic restorations provide an initiative to overcome these problems.

1.3 All-Ceramic Restorations

Ceramics play a vital role in dentistry, with the introduction of the first all-porcelain "jacket" crown dating back to 1889. These crowns were used extensively until the 1950s, however, micro cracking became an unavoidable issue and therefore alternatives needed to be produced. The 1980s saw the development of optimal pressable glass using new production techniques. This later led to the advancement of computer aided design and computer aided manufacture technology, and the birth of a new era in restorative dentistry (Helvey, 2018.,). Monolithic alternatives to the existing layered ceramic restorations provided many benefits for the patients and clinicians. Materials that may be used as part of all-ceramic restorations include glass ceramics such as lithium disilicate and zirconia. Different circumstances in cases may also require the use of multiple ceramics for the same restoration to achieve the desirable characterises, for example lithium disilicate layered on a zirconium oxide framework. Monolithic and veneering production techniques present different advantages and disadvantages, which need to be considered thoroughly (Edelhoff and Brix, 2011.,).

1.3.1 Glass-Ceramics

Glass ceramics have been widely used in medicine and dentistry due to the ease of their processing and the mechanical properties they present. These ceramics show good colour stability, high strength values, chemical resistance and biocompatibility.

Glass-ceramic materials are polycrystalline solids containing a residual glass phase, formed when glass is melted, and then converted into products under controlled crystallization. This process separates the small glass crystals from the glassy parent phase. Heat is used to control the quantity, size and rate of the crystals growth. To ensure enough strength in the glass-ceramic, the crystals must be numerous and uniformly distributed within the matrix. Glass-ceramics in dentistry can be castable, pressable, machinable and can come in an infiltrated state. (Hussain and Santos, 2013.,).

Lithium disilicate based glass ceramics were developed due to their increased fracture resistance and greater strength compared to leucite type glass-ceramics. Controlled nucleation and crystallization enabled lithium disilicate to be developed under the SiO_2-Li_2O-K_2O-ZnO-P_2O_5-Al_2O_3 system. With a high crystalline content of up to 70%, the crystals measure 3 to 6µm in length. The material exhibits a mechanical strength of 350MPa and fracture toughness of 2.5Mpa x $m^{1/2}$. Figure 1.3.1.1.1 shows the needle-like crystals which make up the micro-structure (Ritzbereger et al, 2010.,).

1.3.1.1 Structure and Manufacturing of glass-ceramics

Glass ceramics can be classified by their microstructure where they are defined by the composition of the glass-to-crystalline ratio. Lithium disiillicate which was introduced by Ivoclar as IPS e.max®, is a category 2 ceramic due to the varying amount of crystals added in the matrix, including lithium oxide. This was developed to increase the mechanical strength, opaque qualities, durability, biocompatibility and chipping resistance of the material (Dolidze and Bitarova, 2016.,). Lithium disilicate ceramics also provide exceptional aesthetic properties without the need for additional veneering ceramics, which leads to greater structural integrity and lower rates of fracture and failure (Elsaka and Elnaghy, 2016.,).

Fig 1.3.1.1.1 Microstructure of IPS e.max

Press (SEM) (Denry and Holloway, 2010.,)

Glass ceramics are formed during controlled heat treatment where crystallisation occurs, with the nucleation and growth of internal crystals. This allows the process of ceraming to occur where the structure changes from glass to partially crystalline. Thus, a glass ceramic is a multiphase solid containing a residual glass phase with a finely dispersed crystalline phase (McMillan, 1964.,). The size of the crystal growth is regulated by the temperature and time taken during the ceraming process. This is composed of 2 main stages; glass is heated up to a temperature where nuclei form (750°–850°C), so that nucleation can occur. The temperature is then raised to 1000º-1120ºC for 1-6 hours to allow an acceptable amount of glazing to occur, and crystallization to be completed (Shenoy and Shenoy, 2010.,). Research by Holand et al (2006), concluded that the processes of nucleation and crystallization are vital for controlling the properties and reactions of the glass ceramics; therefore, temperature must be regulated accurately. Chemical durability is an essential characteristic dental material must exhibit to be accepted. Anusavice (1992) found that glass ceramics are among the most durable that can be used in the oral cavity.

The values of chemical solubility for IPS e.max press from his experiment are presented in table 1.3.1.1.1.

Table 1.3.1.1.1. Chemical solubility and limit value of IPS e.max Press

	Chemical solubility [µg/cm²]	Limit value [µg/cm²]
IPS e.max Press	40 ± 10	10

1.3.1.2 High Translucency Glass-Ceramics

The recent development of high-translucency lithium disilicate glass-ceramics has been to improve the aesthetics and appearance of the material for use in anterior restorations. The light transmittance of commercial lithium disilicate glass-ceramics is no more than 10% (λ = 550 nm, d = 0.8 mm), giving it relatively poor optical properties (Llie and Hickel, 2008.,). The translucency depends on the scattering of light which is determined by reflective index and crystalline size. Heffernan et al (2002) found materials composed of small crystals (~ 100nm) showed greater amounts of light translucency then larger lithium disilicate crystals, which was 0.5-4µm. To develop high-translucency lithium disilicate, two stage heat treatments were used on Li_2O-SiO_2-Al_2O_3-K_2O-P_2O_5 glass systems. This process resulted in more effective nucleation leading to crystals of the optimum size. The real time transmission of this was measured at 27.3%, which is far greater than that of commercial lithium disilicate (Bai et al, 2017.,).

1.3.2 Zirconia

Zirconia is a crystalline dioxide of zirconium, which was first introduced to the medical sector in 1969 for orthopaedic purposes. This was an ideal replacement for titanium and alumina in hip head replacements. With an increasing occurrence of allergic reactions and cytotoxicity from alloys, dentists wanted to adopt a material with high aesthetics and safe biological properties (Madfa et al, 2014). The use of zirconia in

dentistry has become highly popular due to its superior toughness, biocompatibility and fatigue resistance. The most common form is a modified yttria (Y_2O_3) tetragonal zirconia polycrystal (Y-TZP). Yttria is added to the composition to stabilise the crystal structure and enhance the physical and mechanical properties of the final product (Bona et al, 2015). Although the characteristics of lithium disilicate and zirconia restorations are similar, key differences can alter the selection of the material which may be selected for certain patients. Zirconia has a greater level of opacity which makes it ideal for darker preparations, although some researchers argue this reduces the aesthetic value.

1.3.2.1 Structure and Manufacturing of zirconia

Zirconia is also more frequently used for posterior prostheses due to increased flexural strength and a high crystalline structure, enabling it to withstand the greater occlusal forces (Tuncel et al, 2016). Y-TZP based ceramics are used in dentistry for implants, bridges and fabricating posts. The reinforced mechanism of zirconia is based on the stress-induced phase transformation from the tetragonal to a monoclinic crystal phase. This gives it a biaxial strength value of between 900-1200 Mpa, and a fracture toughness of 4-5 Mpa $m^{1/2}$ (Gladwin and Bagby, 2012). However, the increase in the monoclinic phase causes a reduction in the materials strength and density, potentially leading to micro-cracking. Surface degradation at low temperatures can also induce surface roughening and generation of micro-debris in the oral cavity (Madfa et al, 2014). The comparison of the properties of pre-sintered and sintered ZrO_2 blanks can be seen in Table 1.3.2.1.1. The porosity, hardness and strength of the material can be coordinated with the milling time during the CAD and CAM process. The restoration must then be densley sintered between the temperatures of 1400^0 – 1500^0C, according to the cycles recommended by the furnace manufacturer (Ritzberger et al, 2010.,).

Table 1.3.2.1.1. Pre-sintered and post-sintering properties of zirconia (Ritzberger et al, 2010.,)

Pre-sintered ZrO_2		Final dense sintered ZrO_2	
Properties		**Properties**	
Density (g cm^{-3})	3.09–3.21	**Density (g cm^{-3})**	>6.0
Porosity (%)	47.3–49.3	**Porosity (%)**	<0.5
Biaxial flexural strength (Mpa)	50–90	**Biaxial flexural strength (Mpa)**	>900
ZrO_2 (Wt %)	87.0–95.0	**Fracture toughness, KIC (MPa m1/2)**	5.5
$Y2O_3$ (Wt %)	4.0–6.0	**Hardness HV10 (Mpa)**	13000
HfO_2 (Wt %)	1.0–5.0	**CTE(100–400°C) (10-6 K-1)**	10.75
Al_2O_3 (Wt %)	0.1–1.0	**CTE(100–500°C) (10-6 K-1)**	10.8

Sintering is the process of compacting compounds into a solid mass under heat and pressure conditions, without melting it to the point of liquefaction. Sintering ceramics at high temperatures fuse the particles together to create a strong and structured material. Fully sintered zirconia has a lower volume of pores, greater values of strength and a larger resistance to hydrothermal ageing. This form can also be milled to the desired dimensions without any shrinkage, however, this does lead to longer milling times and rapid wear of the machining tools. The pre-sintered blocks of zirconia must be sintered again following the milling process, to achieve maximum strength, therefore the sintering shrinkage must be considered before the designing of the restoration. CAD and CAM systems usually utilise these types of blocks due to higher productivity levels, and easier processing (Oh et al, 2010.,).

1.3.2.2 High Translucency zirconia

High translucency zirconia has recently been introduced onto the dental market. This is produced by chemically and physically reprocessing zirconia to reduce the particle size and then shaped through a unique process. Research has found that depending on the sintering condition of Y-TZP, ceramics can be made more translucent while retaining their strength values. This is achieved by reducing the time of sintering at the optimal temperature. High translucency monolithic zirconia materials including BruxZir® provide good aesthetics like those of natural human dentition. The manufacturers of Lava Plus zirconium oxide materials have reduced the quantity of aluminium in their zirconia to 0.1%, leading to reduced light scattering and increased the translucency, which was determined using a dental spectrophotometer (Church et al, 2016.,).

1.4 Surface Characterisations

Natural tooth colour depends on the structure of the enamel and dentine. Enamel is translucent and opalescent due to the hydroxyapatite crystals that form enamel rods. Opalescence leads to a blue and orange colour formation, depending on the interaction of light with the dentition. These colours can be introduced in restorations using internal and external staining. Enamel is also the hardest mineralized tissue in the human body; however, it faces the challenge of maintaining its integrity with constant demineralization in the oral cavity, as it is unable to regenerate. Excessive wear of enamel can expose the darker underlying dentine, resulting in an unaesthetic appearance (Jayasudha et al, 2014.,).

Dentine consists of inorganic components such as hydroxyapatite crystals, which promote light scattering leading to a varied quantity of translucency. Gingival areas have a thin layer of enamel and a greater amount of dentine; thus, the area demonstrates

darker properties when shade matching. Porcelain layering on restorations must mimic the correct relationship between the enamel and dentine thicknesses. External shading materials can therefore be used on restorations to mimic darker areas of the teeth (Yusa, 2009.,).

1.4.1 Dental Glaze

Glazing of dental ceramic materials is traditionally done using two methods, auto or over-glaze techniques with compatible low fusing ceramics. This process is conducted to achieve an impervious surface with added strength due to the glazes' ability to reduce surface flaws. Small surface imperfections and fractures are removed when the glaze is fused to the underlying ceramic sub-structure (Cattell et al, 2009.,). This also provides greater hygiene for restorations as plaque accumulation on the seamless surface is reduced. Glazed ceramics also lead to high lustre which mimic natural dentition as this replicates the appearance of adjacent teeth.

The composition of dental glazes consists of alkali aluminosillicate glass. The glass is embedded within a matrix which allows easier application of the product. During the glazing cycle in a ceramic furnace, the particles of the materials melt and fuse to the surface of the ceramic. This occurs at a lower temperature then the enamel and dentine firings to prevent the deformation of the restoration. Unglazed and un-trimmed porcelain may also lead to the inflammation of the soft tissues it contacts, due to increased surface texture and indentation. This may lead to future problems as gingival tissue is fragile and may become irritated from recurring contact (Al-Wahadni and Martin, 1998.,).

An in-*Vitro* investigation by Jagger and Harrison, found the rate of enamel wear was similar against glazed and un-glazed porcelain. The study was conducted with the use of a chewing simulator to imitate the natural behaviour of the dentition. The results

highlighted the potential damage the surface glazing can inflict on natural tooth structures by causing demineralisation. This can also lead to the dispersion of ions from the glaze and enamel into the oral environment, which may cause cytotoxic reactions.

Azogui et al. (2013) analysed the properties of lithium disilicate glass ceramics following different glazing and polishing methods, in a physio-chemical and biological study. The surface characterisations from glazing and polishing the ceramics were analysed by water-drop methods, interferometry and scanning electron microscopy. It was deduced that the glazed ceramic had worse proliferation and adhesion than polished ceramics. This therefore suggests glaze particles are released into the oral cavity, due to reduced amount of retention of the material on the ceramics surface. The study also reinforced that lithium disilicate glass ceramics are not cytotoxic and are inert when used for dental restorations.

1.4.2 External Dental Stains and Shades

The use of dental stains and shade colours enhances the three-dimensional nature of the restoration to produce natural looking ceramic prostheses. Colours may be mixed to reproduce the natural aesthetics of adjacent teeth and reduce the prominence of the appliance in the mouth. Application of a thin layer of stain can have a big impact on the final colour following the firing process. Dental Stains are commonly opaque with a high colour peak emission, which can easily lead to an uneven consistency during the application process. The paste can be mixed with a dilution liquid to achieve the desired viscosity, resulting in an even application. If the mixture is too viscous, the correct intensity of shade may not be achieved (Yusa, 2009.,). Furthermore, external stains increase light absorption and reduce the translucency and opalescence that can be created in the ceramic restoration. This can be a drawback for anterior restorations as light transmission

is reduced. To overcome this, the further addition of opalescent and fluorescent ceramics can help re-establish the natural characteristics (McCabe and Walla, 2008.,).

Multiple layering and firing cycles can be used to produce the correct colour for the ceramic restoration. The 1st layer of shade paste is applied to achieve the correct basic shade, until the proper hue, chroma and value is matched. Special effects and colour can also be added in the final stain firing. These include the use of blue colours on the incisal edges of the crown and orange-based colours placed closer to the cervical areas. Following all the staining procedures, glaze can then be applied and fired according to the parameters provided by the company.

Dental shading materials are essentially made up of metal oxide particles, which may cause increased wear of the ceramics, as they are abrasive to enamel. This can also lead to the suspension of ions the mouth following the breakdown of the ceramic or stains, potentially resulting in cytotoxic reactions. This issue can be overcome with the use of glazes over the stains and shades to reduce the amount of oxide ions exposed and released into the oral environment (Daou, 2015.,). As a result of this, external staining is less desirable than internal stains as it may wear off over time, during the ageing process.

1.5 Biocompatibility

Dental devices and materials must undergo rigorous testing to ensure they are safe and biocompatible in the oral environment. Biocompatibility is the ability of a biomaterial to co-exist with tissues and carry out the desired function without inflicting any local or systemic effects. Some dental materials may have adverse effects on tissues and physiological systems, depending on their properties or the sensitivity of the patient. A material must only be described as non-biocompatible if it elicits specific bodily responses when tested with a specific tissue under certain conditions (Williams, 2008.,).

The conditions of the mouth can also impact the level of biocompatibility which is achieved by the material. Exposure to saliva, bacteria and foodstuffs can influence and alter the behaviour of the materials in the mouth. Extreme thermal changes in the oral cavity may lead to thermal expansion of materials. These fluctuations can lead to the chemical and mechanical degradation of materials. The reflux of gastric contents with a pH of 0.8, may also cause degradation of materials due to the exposure to acidity. Masticatory forces over extended time periods also contribute to the continuous and progressive degradation of the surface characterisation materials (Gupta et al, 2012.,).

1.5.1 Cytotoxicity of Dental Ceramics

Cytotoxicity is the destruction of cells caused by chemicals or materials. This process inhibits the growth of the cells in contact with the source of the specific material. Cytotoxicity can be estimated using cell proliferation and DNA synthesis. Messer et al evaluated the cytotoxicity of all-ceramic materials using a cell culture generated from the mouse fibroblast. The results reported that IPS e.max and zirconia demonstrated significantly higher toxicity levels ($P<0.05$) when compared to other feldspathic ceramics. This research contradicts the previous studies carried out by Azogui et al, which suggested that lithium disilicate was safe to use in the oral cavity. However, the cytotoxicity level reduced significantly following the ageing process of the ceramic, potentially because of excessive wear reducing the number of cytotoxic particles and ions (Kilic et al, 2013.,).

Such destructive action, particularly in reference to lysis of cells, selectively kills dividing cells. Testing cytotoxicity is essential to identify compounds, which could potentially harm living tissue**.** Cellular toxicity testing is covered by the International Standards Organisation under ISO 10993-5. This standard covers a number of testing

methods to analyse the biological effects certain materials have on tissue. Cytotoxicity determination can be grouped into categories including measurement of cell growth, measurement of cell damage, and cell damage by morphological means (Wallin, 1998). This standard also states that cell lines other than established or commercially produced ones can be used in cytotoxicity testing (Gociu et al, 2013.,).

1.5.2 Cytotoxicity of stains and glazes

The glaze layer of dental restorations is lost after a short-term period due to occlusal movements and adjustments, even though it has been thought to reduce the wear on antagonist dentition. Kontos et al (2013) used a chewing simulator to prove that sandblasted, ground and glazed zirconia showed the greatest amount of wear, which translates to the suspension of oxide ions in the oral cavity.

However, Yang et al found heating the Zirkonzahn Y-TZP zirconia material showed fewer abrasive qualities without glazing, as opposed to being glazed and stained (Daou, 2015). This therefore suggests that unglazed zirconia leads to less suspension of ions in the mouth, reducing potential cytotoxic effects.

Further research around this subject was conducted by Janyavula et al (2013). They found polished zirconia demonstrated significantly less wear then glazed zirconia. The initial surface roughness presented by the glazed ceramics is also greater leading to a higher friction co-efficient. This could lead to a greater concentration of glaze components suspended in the oral cavity, which may lead to potential cytotoxic results over-time. During wear tests, the 20 to 50 μm thick layers of glaze on the specimens were worn away.

The changes in pH of the oral cavity can also influence the ionic release from dental ceramics and surface characterisation materials. This can be caused by dietary

factors or the buffering capacity of the individual's saliva which can alter the chemical solubility of the ceramics. Results from a study found at pH 10, the ionic release of Ca, Li, Zn, and Si was the greatest from the glaze. The over glazes degrade by ion exchange of protons for alkali ions when in an acidic environment. The findings concluded that ceramic glazes are susceptible to degradation in low and high pH environments (Esquivel-Upshaw et al, 2013.,).

1.5.3 Causes of cytotoxicity

The degradability of dental ceramics can occur due to mechanical forces or chemical reactions, causing the potential release of ceramics or ceramic surface treatments into the mouth. Furthermore, some ions which would be less biocompatible, also risk contacting the gingival tissues if excessive abrasion occurs, resulting in cytotoxic effects on cells (Anusavice, 1992.,). Chemical and physical destruction of dental materials can also be caused by saliva, wear, erosion caused by food, chewing and bacterial activity, therefore it is important to test the reactivity of materials in the oral environment. This can be governed by electro-chemical reactions, kinetics and thermo-dynamic principles. During the equilibrium phase of the material, it is neither stable nor releases any ions. This causes the initially uncharged molecules to lose electrons and become positively charged, as they are released into the saliva. This biodegradation of the material reduces the biocompatibility as surrounding tissues are being affected by the on-going ionic release (Elshahawy and Kramer, 2014.,).

Research by Elshahawy and Kramer (2014) analysed the elements released from gold and ceramic dental crowns, which were Au, Zn, Cu, Al and Si. Following an incubation period of 7 days of the ionic salt solution on mouse fibroblasts, the results demonstrated the Zn and Cu produced the most cytotoxic effects on the fibroblast cell

cultures. However, the concentration of these ions was minimal, suggesting there would be no immediate cytotoxic results.

Lee et al (2014) tested the wear of tooth enamel against ceramic and gold alloy restorations. The findings suggested a higher friction coefficient and worse wear resistance was observed against the ceramic restoration. This is a limitation for the use of all-ceramic systems in dentistry. Mine et al (2003) determined the elemental composition of substances lost following wear of all-ceramic dental materials. The study was to evaluate the possible release of ions in the oral cavity with the use of artificial saliva and occlusal wear machine. The statistical analysis revealed no significant difference in Na, K, Ca or Mg levels in leucite-reinforced ceramics, however, there was increased Li suspension in the saliva from lithium disilicate based groups. This suggests Lithia based materials are prone to elemental release over short-term tests.

1.6 Computer Aided Design and Manufacture (CAD/CAM)

A mixture of conventional and modern production techniques can provide benefits and drawbacks for this experiment. CAD/CAM production of the samples can be easy and minimise human error in ensuring the sizes are consistent.

1.6.1 CAD/CAM in Dentistry

The use of Digital technology has recently increased in popularity for the medical and dental sectors. Computer Aided Design and Computer Aided Manufacture is not only available chair side for intra-oral scanning, but also in laboratories for the manufacturing of complex appliances and dental models. Achieving successful restorations requires the use of appropriate software and sufficient expertise of the operator (Tapie at all, 2015.,). The first system was introduced in 1971, however it was not used due to reduced

accuracy and limitations of computer systems. Benefits of the technology leads to increased laboratory efficiency, mechanical durability and predictability. However, the costs and availability of the systems must be considered before the clinical use.

1.6.1.1 Data Acquisition

Dimensions of prepared teeth and related structures can be scanned using conventionally produced models or recently introduced intra-oral scanners. This can be conducted with the use of contact scanners, optical scanners, 3D microtomography (MCT) and nuclear magnetic resonance (NMR). Optical scanners use sensors to capture the patterns of light and shade projected. The receptors interpret changes in depth to establish distances, which generates three-dimensional images (Samra et al, 2016.,).

1.6.1.2 Restoration Design

The images are transferred to a data processing centre where the designing process of the prosthetic infrastructure can commence. A wide range of CAD programmes are available for the manipulation of the images to detect tooth preparations and allow planning of tooth support while considering strength and aesthetics. Undercuts can also be blocked out and die-spacer is applied to imitate space for cementation. When partially sintered zirconia-based materials are used, the design is enlarged by 20% to overcome shrinkage following firing. Whereas, fully sintered zirconia does not encounter this problem when milled and exhibits no changes in dimensions (Koutayas et al, 2009.,).

1.6.1.3 Manufacture

The data produced by the CAD software can be transformed into the physical version through the milling device. Closed systems which were originally used can perform each

stage of the process in a sequential and inter-related manner. However, laboratories which acquired this system would be limited to the use of a single material provided by the manufacturer. The use of open systems allows the data acquisition document to be sent to any data processing centre for designing and milling. The subtractive milling process uses a computerised milling machine containing the required data. The machine shapes the material into the size and detail as designed in the CAD software. The procedure is performed with the use of carbide or diamond burrs in a dry or water coolant environment (Samra et al, 2016). Dry processing is mainly applied to zirconium oxide blanks, which offer benefits including reduced costs for investments in milling devices and no drying time is needed for the ZrO_2 prior to sintering. However, one of the disadvantages of this technique is the Increased amount of shrinkage post-sintering may impact the accuracy of the dimensions of the restorations (Beuer et al, 2008.,).

Wet milling is used for metals, glass ceramics and zirconium oxide. This results in less shrinkage following sintering ultimately preventing sintering distortion. The spray of cooling liquid protects the cutters from overheating and altering the properties of the material. The path of cutting is an essential consideration, especially during the processing of zirconia restorations. Trace lines combined with micro cracks in this material can limit the survival rates of the restorations (Samra et al, 2016.,).

1.7 Lactate-Dehydrogenase (LDH) cytotoxicity assay

Lactate dehydrogenase is a cytosolic enzyme in cells, which is released extracellularly upon cell membrane damage following cell death. The LDH serum levels are raised under pathologic conditions. The four-unit mechanism of this protein is made of single poly-peptide chains, each with the molecular weight of 35,000 subunit (Kleinsmith et al, 2008.,). The enzyme catalyses the conversion of lactate to pyruvate to

terminate glycolysis, in the presence of the coenzyme nicotinamide adenine dinucleotide (NAD) (Adams et al, 1970). This can be seen in Figure 1.7.1. Studies have demonstrated elevated levels of the enzyme are present in the oral cavity and this is associated with gingival inflammation and tissue destruction (Alfaqeeh and Anil, 2011.,). The LDH assay allows the quantity of the enzyme released from the damaged cells to be measured, as an indicator for cytolysis and cytotoxicity. A spectrophotometer is used to access light absorbance from flat-bottomed clear 96-well plates.

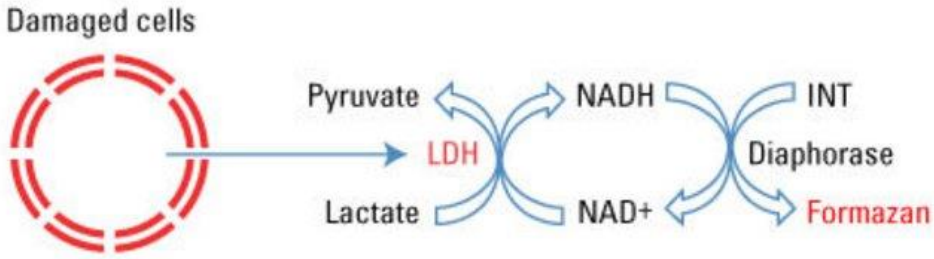

Figure 1.7.1 The basis of LDH cytotoxicity assay (Thermo Fisher Scientific, 2014)

1.8 The impact of cytotoxicity on the oral environment

Studies into the impact of cytotoxicity in the oral environment are essential to gather an understanding of the possible consequences of not dealing with the problem. Dental materials including denture base acrylic resins and bonding agents have been proven to harm teeth and surrounding tissues through toxicology testing. Tests which recreate i*n vivo* conditions provide clinically relevant results (Lee et al, 2017.,). This is the reason why the impact of surface characterisation materials must be assessed to gain an understanding of their potential effects in the oral environment.

The location of the material in the oral cavity can influence the type of reaction caused by the lack of biocompatibility. Dental glaze, stains and shades are exposed to soft tissues, saliva and other fluids depending on their placement internally or externally to the oral epithelium. Furthermore, the duration the material is in the oral environment

can impact the levels of bodily responses as interactions between the body and materials take time to develop (Perrotti et al, 2017.,). The possible toxic effects to human oral epithelium from dental materials may be caused by the dissolution of chemical and ionic components which implement local systemic and carcinogenic effects. Many studies have demonstrated the long-term effects of nickel-ion release from dental materials on oral tissues. The results from research by Trombetta and Saija (2005) reinforced the assumptions that cytotoxicity caused by the metal alloy causes changes to cellular functions and morphology. However, the underlying causes of the nickel toxicity remain uncertain.

Analysis of literature by Atai and Atai (2007) demonstrated the complications which can occur due to reduced biocompatibility of a dental material in the oral cavity. Diffusible toxic substances from materials can be led into the circulatory system, resulting in a systemic toxic reaction. Also, the oral mucosa may display lichenoid lesions because of contact with metallic components in materials. However, this can be treated following the removal of the dental appliance. Many materials have been proven to cause allergic stomatitis including mercury present in amalgam and monomer in acrylic denture base resins. The aesthetic, mechanical and physical properties of dental materials and products can unexpectedly cause many reactions and issues in the oral enviroment, therefore the exploration into characterisation materials must be thorough.

1.9 Aims and research hypothesis

There are many pieces of research available on the properties, compositions and cytotoxicity of dental ceramic materials. This presents a valuable insight into the impact of materials on cell activity. The cytotoxic effects of dental surface characterisations require further investigation. The limited research regarding this topic does not provide

evidence to prevent the use of the materials on dental ceramics. With these materials being used daily for millions of patients, their impact needs to be investigated thoroughly to ensure no harm is being induced on the individual's health or well-being.

The aim of this experiment is to establish any possible cytotoxicity caused by the application of extrinsic glaze and shades on all-ceramics. Based on previous experimental studies and literature reviews, the expected results for this study should show no significant difference in cytotoxicity of samples, which have and have not had surface characterisations applied. This is due to the lack of research present on any potential toxicity of the materials, and the reassurance provided by manufacturers.

2.0 Materials and Methods

2.1 Specimen fabrication

2.1.1 Glass ceramic specimens

Specimens for the glass ceramic materials were designed using the Solid-works software (SolidWorks, Massachusetts, USA) and milled (DWX-52DCi, Roland, Osaka, Japan). These had the dimensions of 9mm x 6mm x 1mm, which are in accordance with ISO 10993-5. The samples were milled using wax milling discs (ZirLux, Langen, Germany).

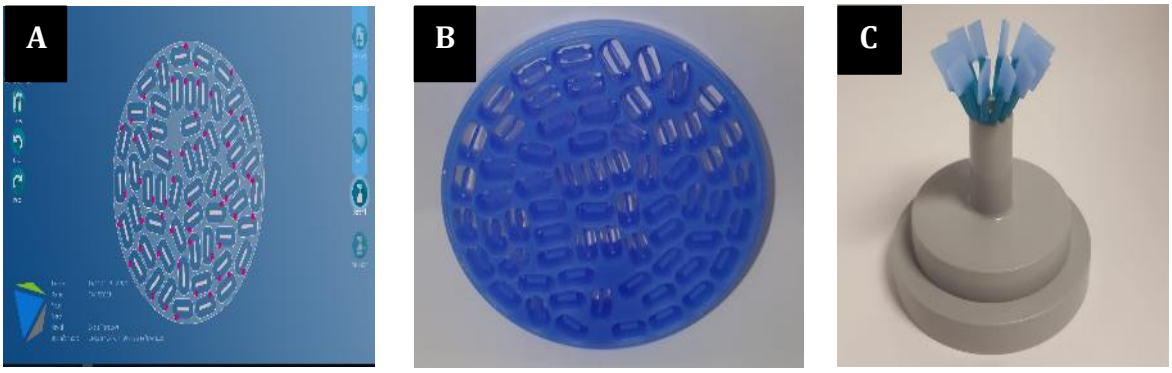

Figure 2.1.1 **A)** CAD design of specimens **B)** Milled was disc **C)** Sprued specimens ready for investment

The samples were then sprued using oxide-free sprue wax. This was done in accordance to the manufacture's guidelines, with the sprues having a height of 8mm and the investment ring having a total weight of 1.7g, to ensure successful pressing with a 200g ceramic ingot. Ivoclar PressVest Premium investment powder and expansion liquid were used at the ratio of 36ml to 16ml of distilled water. A setting time of 20 minute for the investment was followed by the insertion into a wax burning out furnace (Carbolite gero, Derbyshire, UK) at 850^{o}C for 60 minutes, which removed all the waxy residue. This

procedure was repeated for the Celtra ceramic specimens, with the compatible investment powders and expansion liquid as stated by the manufacturer. This was left to set for 20 minutes and was then inserted into the burn-out furnace at 850 degrees, at an angle of 45^0. Once the burnout process was complete, the investment ring was transported into the pressing furnace.

The Ivoclar Programat EP 3010 furnace (Ivoclar Vivadent, Schaan, Liechtenstein) was used for the pressing of the ceramic ingots. Low translucency, shade A3 IPS e.max press ingots for lithium disilicate were used. For the Celtra group, low translucency shade A3 ingots were used.

The heat pressing programmes for a 200g muffle for the Celtra test group are presented in table 2.1.1:

Table 2.1.1 Heat pressing programme for Celtra specimens

Heat Pressing Programme	Celtra
Start Temp (°C)	700
Heating rate (°C/min)	40
Final Temp (°C)	865
Hold Before Pressing (min)	30

Manufacturer's instructions according to Ivoclar Vivadent were followed for the pressing of the 200g e.max samples. For the e.max specimens, alumni oxide plungers were submerged in a boron nitride separator and placed above the ingot and into the furnace. Celtra press investment plungers (Dentsply Sirona, York, Pennsylvania, USA) were used for the pressing of the Celtra specimens to lower the ingot into the mould.

Lithium disilicate and Celtra specimens were divested (BEGO, Florida, USA) carefully at a pressure of 2.0 bars using 50-micron alumni oxide beads, however this was reduced to 1.5 bar for fine divestment. This was performed by a single person at 10mm at a 45^{o}C angle. The ceramic sprues were removed using a diamond cut off disk 2mm away from the edge of the specimens at 10000rpm. Trimming stones (Shofu Inc, Kyoto, Japan) were used to remove the excess ceramic from the sprue attachment area. Following this, the specimens were steam cleaned (Amann Girrbach, Koblach, Austria) for 30 seconds and air dried (Ozdemir and Aladag, 2017.,).

A metallographic grinding machine (Struers, Ballerup, Denmark) was used to grind all the surface of the glass ceramic specimens. This was conducted using P500 silicon carbide disc paper (Struers, Ballerup, Denmark) and the gentle flow of water, which was replaced after every 5 specimens (Brackett et al. 2008). The specimens were then lightly abraded with 50-micon alumni oxide at a pressure 1.5bars.

The samples were then cleaned ultrasonically (Walker electronics limited, Alton, United Kingdom) for 5 minutes using distilled water to remove potential contamination of the surface (Bottino et al, 2006). These were then steam cleaned, air dried and stored in an air tight petri-dish until the glaze and shades were applied.

2.1.2 Zirconia specimens

KATANA zirconia (Kuraray, Sakazu, Japan) was used to produce the zirconium specimens. Test samples were designed on the solid-works software with the same dimensions of 9mm x 6mm x 1mm and dry milled according to the milling systems technical guide. Carbide burrs were used to detach the specimens from the milled disc

and remove the excess attachments. These were then sintered at the temperature of 1550^{o}C for 2 hours (Kavo Dental, Biberach, Germany).

Following the sintering procedures, the samples were sandblasted using 50-micron alumni oxide at a pressure of 1.5bars. Ultrasonic cleaning was conducted using distilled water for 5 minutes. Before the glazing and staining procedure, all the specimens were steam-cleaned thoroughly and dried.

Figure 2.1.2.1 **A)** Milled Zirconia Specimens **B)** Preparation for sintering **C)** Sintering at 1550^{0}C

2.2 Surface characterisation material

2.2.1 Scoop Design and Manufacture

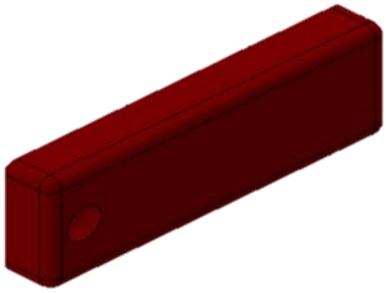

Figure. 2.2.1.1 Digital scoop design to be milled in

Custom zirconia scoops with the volume of 14.1mm^3 and 150.4mm^3 were designed using the Solid-works software. These were milled and sintered using zirconia discs (ZirLux, Langen, Germany) to measure the surface characterisation materials.

2.2.2 Preparation of glaze and shades

A wide range of ceramic shades and glazes were used for this experiment, each were compatible with the relevant protocols and instructions provided by the manufacturers. The full variety of glaze and shades used are presented in table 2.2.2.1. IPS Ivocolor shades were used for the lithium disilicate specimens. The glaze material used for samples was the IPS Ivocolor glaze paste, which was mixed with 1 drop of the all-round Ivocolor mixing liquid. For the Celtra samples, Dentsply Sirona shades were used, alongside the universal stain and glaze liquid. All shades and glazes were mixed with an Agate spatula (Renfert, Hilzingen, Germany) to ensure an even consistency and distribution of pigments, without the contamination of metallic ions. A ceramic mixing tray (Renfert, Hilzingen, Germany) was used to prevent potential contamination of extraneous minerals. The process of preparing the glaze and shades can be seen in figure 2.2.2.1.

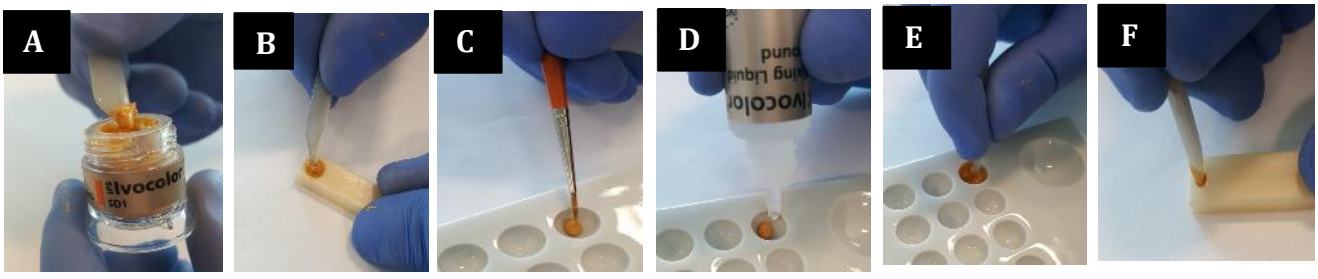

Fig. 2.2.2.1 **A)** Mixing the shade **B)** Measuring quantity in large scoop **C)** Transferring to ceramic mixing tray **D)** Adding the mixing liquid **E)** Mixing to achieve correct consistency **F)** Transferring to small scoop for induvial sample

Table 2.2.2.1 Glazes, shades and mixing liquids for all 3-ceramics tested

Characterisation material	*E. max IPS.*	*Celtra*	*KATANA*
Shade A	SD1 (3g)	BODY S1 (5g)	Cerabien A+
Shade B	SD6 (3g)	BODY S4 (5g)	Cerabien D+
Glaze	Ivocolor glaze paste (9g)	HIGH FLU overglaze (5g)	Cerabien clear glaze
Mixing Liquid	All- round Ivocolor mixing liquid (15ml)	Universal stain and glaze liquid (15ml)	

The shades were transferred into the large scoop and vibrated with the rough edge of a tool. The excess stain and glazes were removed using the flat edge of the smaller scoop. The shade was then transferred into the ceramic mixing tray and 1 drop of mixing liquid for the glass ceramic specimens was added to achieve the correct consistency of material. The KATANA specimens did not require a mixing liquid, and therefore the stains and glaze were directly transferred into the small. The group names and sample sizes for all materials can be seen in table 2.2.2.2.

Table 2.2.2.2 Groups, number and surface characterisations

Group name	*Number of specimens*	*Surface Characterisation*
A	7	No Treatment (Control group)
B	7	Glaze Only
C	7	Glaze & Shade A (mixed)
D	7	Glaze & Shade A (1 layer)
E	7	Glaze & Shade A (2 layers)
F	7	Glaze & Shade B (mixed)
G	7	Glaze & Shade B (1 layer)
H	7	Glaze & Shade B (2 layers)

2.2.3 Application of glaze and shades

The surface glaze and shades were applied with a small staining brush (Renfert, Hilzingen, Germany) and lightly vibrated using an abrasive tool (Cattell et al, 2009). Rubber tipped zirconia tweezers (Hammacher, Strabe, Germany) were used to transfer the stained and glazed samples to the custom zirconia stands as seen in figure 2.2.3.1, which were placed on a ceramic dental honeycomb firing tray. The 7 zirconia stands were placed in a circular formation, all at an even distance from the centre of the stand. The stands were smoothened and polished using burrs (NTI-Kahla GmbH, Kahla, Germany) between uses. A visual representation of this can be seen in figure 2.3.2. The specimens were fired using the Ivoclar Programat EP 3010 furnace (Ivoclar, Schaan, Liechtenstein) according to the parameters on table 2.2.3.1.

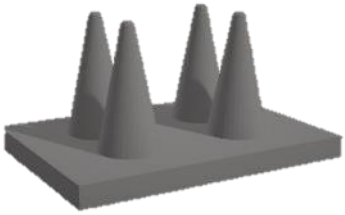

Figure. 2.2.3.1 Digital design of zirconia firing

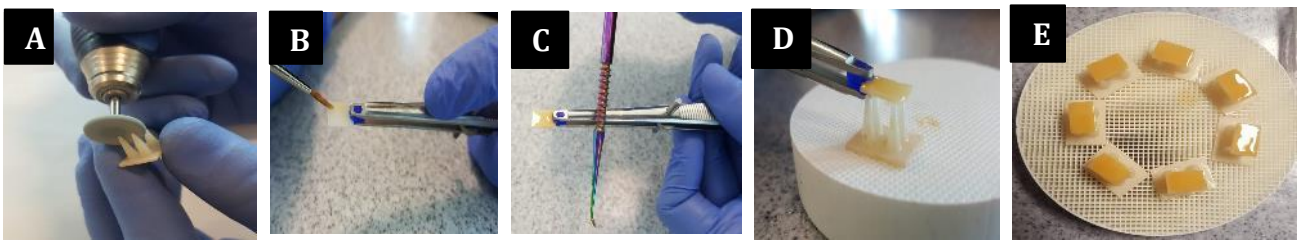

Figure. 2.2.3.2 **A)** Polishing of the zirconia stands **B)** Application of the external glaze and shades **C)** Light Vibration **D)** Placement on the zirconia stand with silicon tweezers **E)** Circular arrangement on firing disc

Table 2.2.3.1 The firing cycle of the materials following staining and glazing

Firing Programme	**IPS e.max Press**	**Celtra**	**Katana**
Firing Temp (°C)	710	760	750
Standby Temp (°C)	403	403	403
Closing Time (min)	6	6	5
Heating Rate (°C/min)	60	55	45
Holding Time (min)	1	2	1
Vacuum 1 (hPa)	450	0	600
Vacuum 2 (hPa)	709	0	750

2.4 Cytotoxicity testing

Before commencing with the cytotoxicity testing, all specimens were sterilised in an autoclave (Tactrol 2, Priorclave, London, UK) at a pressure of 15psi and at a temperature of 120^{0}C for 2 hours.

2.4.1 Media Preparation

5ml of Penicillin (Gibco, ThermoFisher, Hempstead, UK) was added to 500ml of serum-free media (Dulbecco's Modified Eagle's Medium, ATCC, Manassas, USA). The specimens were then added to the 460µL of the prepared media in individual eppendorf tubes and placed in the Hula mixer (ThermoFisher scientific, Hempstead, UK) for 72 hours.

2.4.2 Cell Culture

Human gingival fibroblasts (HGF-1, ATCC, Teddington, UK) were used for the Katana and Celtra, whereas Human dermal fibroblasts (HDF-1, ATCC, Teddington, UK) were used for the e.max pilot study. This cytotoxicity testing and grown in serum-free media for 24 hours in a flask at 37^{0}C. The media was then removed, and the cells were washed with 5ml of sterile phosphate-buffered saline (PBS) (Corning, Tewksbury, United States) and gently vibrated. The PBS was then removed. 5ml of trypsin (ThermoFisher, Hempstead, UK) was added to detach the cells from the surface and incubated (NUAIRE, Doncaster, UK) for 10 minutes at 37^{0}C. Following this, 5ml of serum-free media was transferred into the same flask, and the contents were carefully transported into a test tube. This was then centrifuged for 4 minutes, at 1700rpm to expose a pellet of cells. The trypsin and media were then removed. The pellet containing the cells was re-suspended into fresh serum-free media using a pipette. A haemocytometer was then used to ensure

the cells were suspended in the media and 10µL of the solution was used to count the number of cells present.

1ml of the cells and media were then moved into 2cm^2 wells of a 24 well tray, as seen in figure 2.4.2.1. These were then be incubated for 24 hours at 37^{0}C, to allow the cells to grow. The media was then removed, and the cells were washed with 1ml of PBS. Following this, 420µL of the leached media was inserted into each well. This was then left for a further 24 hours at 37^{0}C.

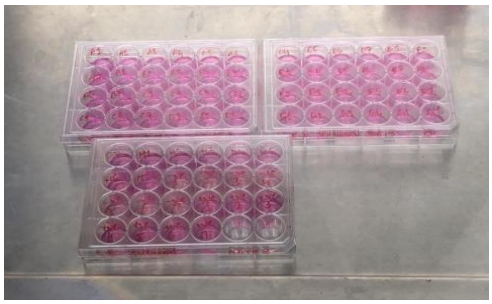

Figure. 2.4.2.1 24-well tray containing human gingival fibroblasts and serum-free media

2.4.3 Reaction mixture preparation

To 1 vial of powder lyophilizate, 11.4ml of ultra-pure water was added and gently vibrated. This was combined with 0.6ml of assay buffer, to gain 12ml of the reaction mixture (ThermoFisher scientific, Hempstead, UK). This was stored in a dark environment until use.

2.4.4 LDH Assay

The media from the positive control wells was removed, and 10µL of lysis buffer (ThermoFisher scientific, Hempstead, UK) was added and lightly mixed to achieve maximum LDH activity. This was left to incubate for 45 minutes. The negative control consisted of un-treated serum-free media. 50µL of the leached media was transferred from one well of the 24 well tray into 3 wells of the 96-well plate (Nunclon, Denmark) using

a pipette (Biopette, Labnet, New-York, USA). 50µL of the reaction mixture was added to all wells and incubated at room temperature. Following 30 minutes from the first addition of the reaction mixture, 50µL of stop solution was added to every well.

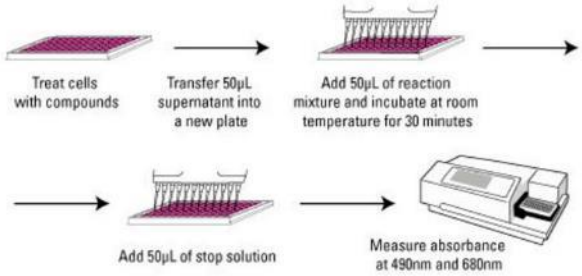

Figure. 2.4.4.1 Protocol for LDH cytotoxicity assay (Thermo Fisher Scientific, 2014)

2.4.5 Measuring light absorbance

The plates 96-well plates were then centrifuged for 6 seconds and the light absorbance was measured with an absorbance reader (Biotek, Winooski, USA) at frequencies of 490nm and 680nm This is demonstrated in figure 2.4.5.1. The calculation for the % of cytotoxicity can be calculated with the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Compound treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}}$$

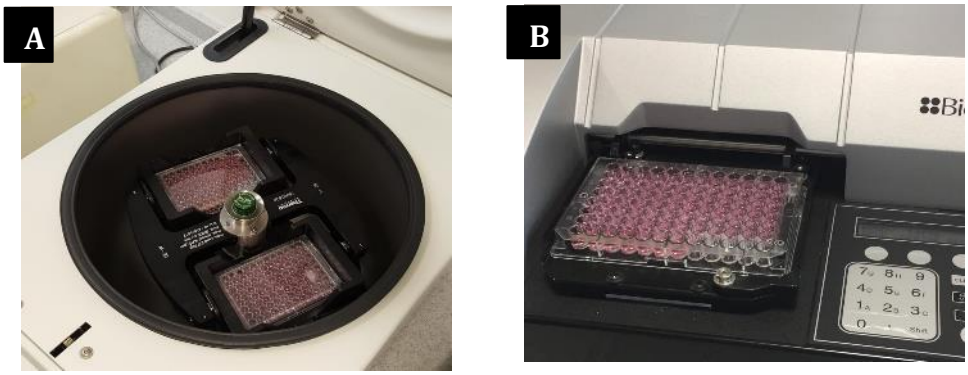

Figure. 2.4.5.1 **A)** Centrifuging of the 96-well plates to remove air bubbles **B)** Spectrophotometer measuring light absorbance at 2 different frequencies

2.5 Statistical analysis

Statistical analysis was conducted using on-way analysis of variance (ANOVA), Tukey's, Shapiro-Wilk, Kolmogorov- Smirnov and Kruskal-Wallis. Normality tests were conducted to determine the significant differences between groups and the Kruskal-Wallis to accept or reject the null hypothesis. A statistical analysis software was used to conduct this (SPSS 23, IBM, New York, USA).

3.0 Results

3.1 e.max

It was found that the glazed specimens showed the least cytotoxic activity compared to all other specimens. On the other hand, the specimens surface treated with glaze and shade A (mixed) showed the highest levels of cytotoxicity. This difference in between these groups was statistically significant (Figure 3.1.1).

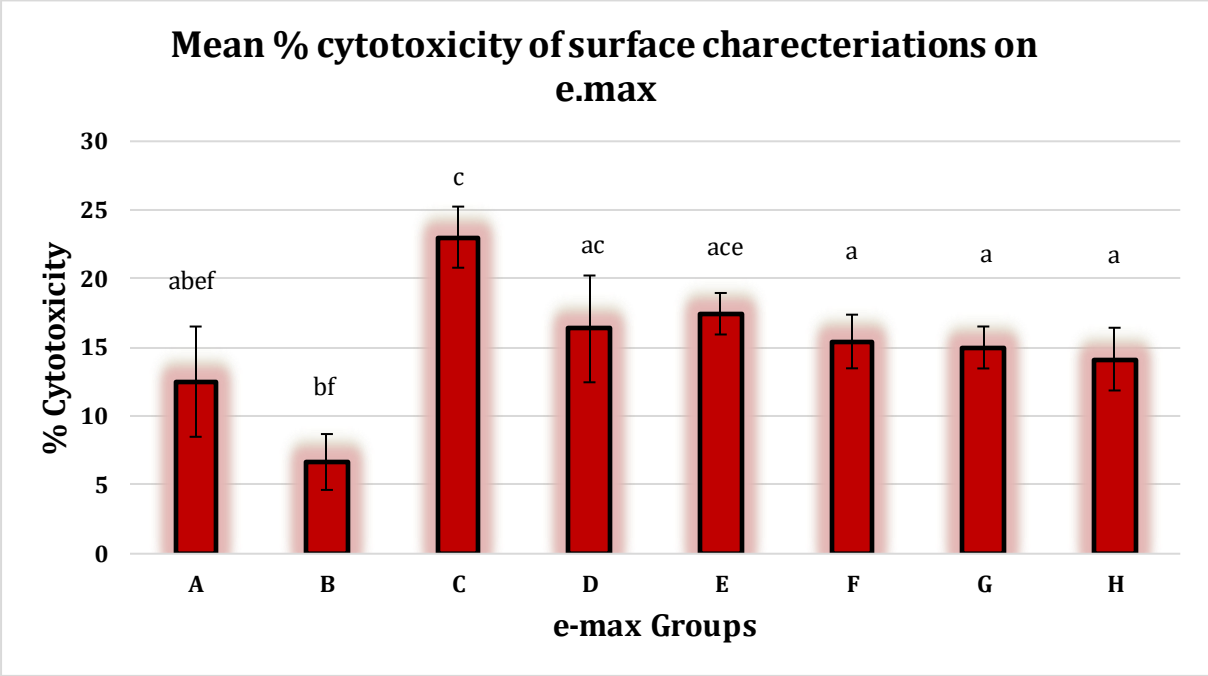

Figure 3.1.1 The mean % cytotoxicity of different surface characterisation groups applied on e.max. Based on cytotoxicity tests using LDH assay on human gingival fibroblasts. This displays the standards deviations and significant differences between the different categories. p=0.5. Refer to table 2.2.1.1 for the description of the groups.
At least on matching letter means no-significant difference between the groups at the value of 0.05.

Table 3.1.1 Numerical values for Figure 3.1.1

Groups	Mean % Cytotoxicity ± (SD)	Significant Difference
A	12.516 ± (4.011)	abef
B	6.672 ± (2.032)	bf
C	23.026 ± (2.223)	c
D	16.354 ± (3.883)	ac
E	17.457 ± (1.509)	ace
F	15.438 ± (1.952)	a
G	15.002 ± (1.525)	a
H	14.156 ± (2.281)	a

3.2 Katana

It was found that group C with glaze and shade A (mixed) showed the greatest cytotoxicity and group H showed no cytotoxicity. The difference between these groups was statistically significant (Figure 3.2.1).

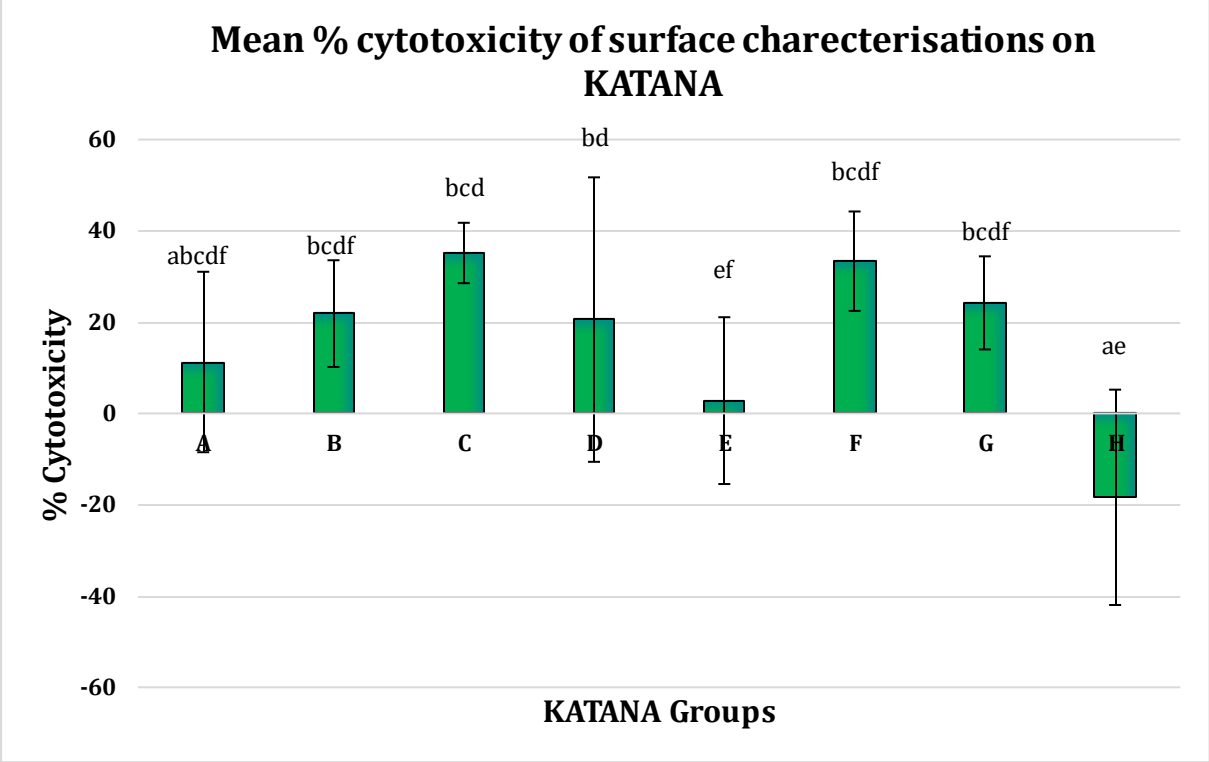

Figure 3.1.2 The mean % cytotoxicity of different surface characterisation groups applied on Katana. Based on cytotoxicity tests using LDH assay on human gingival fibroblasts. This displays the standard deviations and significant differences between the different categories. p=0.5. Refer to table 2.2.1.1 for the description of the groups.
At least on matching letter means no-significant difference between the groups at the value of 0.05.

Table 3.2.1 Numerical values for Figure 3.1.2

Groups	Mean % Cytotoxicity ± (SD)	Significant Difference
A	11.266 ± (19.767)	abcdf
B	21.868 ± (11.690)	bcdf
C	35.148 ± (6.615)	bcd
D	20.556 ± (31.161)	bd
E	2.803 ± (18.273)	ef
F	33.351 ± (10.893)	bcdf
G	24.215 ± (10.200)	bcdf
H	-18.382 ± (23.588)	ae

3.3. Celtra

It was found that the highest level of cytotoxicity was displayed by Group H, which had glaze and 2 layers of shade B. The lowest cytotoxicity was displayed by group B, which only had glaze applied to the surface. There was a statistical difference between these groups (Figure 3.3.1).

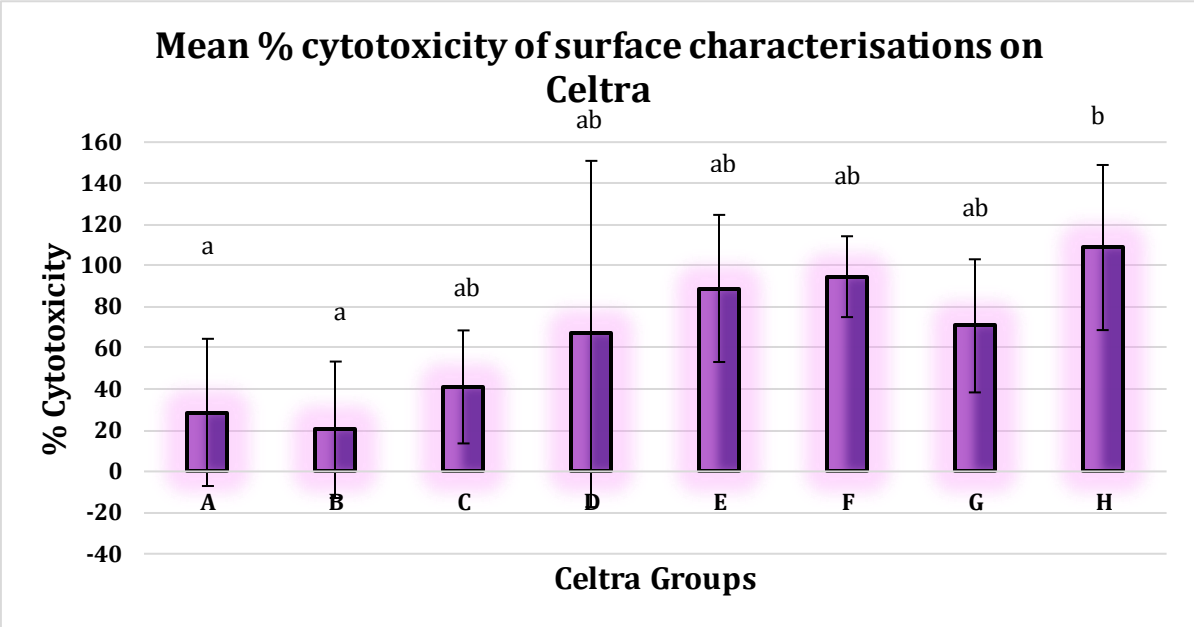

Figure 3.1.3 The mean % cytotoxicity of different surface characterisation groups applied on e.max. Based on cytotoxicity tests using LDH assay on human gingival fibroblasts. This displays the standard deviations and significant differences between the different categories. p=0.5. Refer to table 2.2.1.1 for the description of the groups.
At least on matching letter means no-significant difference between the groups at the value of 0.05.

Table 3.3.1 Numerical values for Figure 3.1.1

Groups	Mean % Cytotoxicity ± (SD)	Significant Difference
A	28.672 ± (35.760)	a
B	20.235 ± (33.156)	a
C	41.061 ± (27.457)	ab
D	66.784 ± (84.121)	ab
E	88.908 ± (35.742)	ab
F	94.631 ± (19.638)	ab
G	70.737 ± (32.345)	ab
H	108.790 ± (40.117)	b

4.0 Discussion

The null hypothesis, that there will be no significant difference in the cytotoxicity of samples, which had and did not have surface characterisations applied, was rejected for the e.max material. This was due to the results having significance level of 0.005, which was lower than the 0.05 threshold, that we set. The reason for the selection of a non-parametric test (Kruskal-Wallis) was due to the sample size being very small. The Tukeys test was selected as more than two groups needed a comparison within the results. The first normality tests were conducted using the Kolmogorov Smirnov test. This was followed by the Shapiro Wilk test which demonstrates greater power for statistical analysis. Both these tests were conducted to increase the reliability of the findings (Ghasemi and Zahediasl, 2012).

The testing of the e.max material was a pilot study, which consisted of 4 specimens per group. The standard deviations for e.max range from 1.509 to 4.011, which are less in comparison to the other materials tested, therefore increasing the reliability of the results. The significant differences for the groups are in accordance with the expected results. For example, no significant difference can be seen between group A which is the control with no treatment and group C which has mixed glaze and shade applied. However, there is a significant difference between B and all other groups. This is due to glaze consisting of glass particles, whereas the other groups contain pigmentations and ions from metal oxides. This increases the credibility of the study as the expected trends are demonstrated.

One-way ANOVA was conducted for the Zirconia specimens (Katana) due to a larger sample size. The cytotoxicity results do not follow a trend based on the surface characterisations applied. This relationship goes against the assumption that surface

glazing and staining causes cytotoxicity within the oral environment. The standard deviation values for the groups A, C, E and H are large, which demonstrates the large range of results gathered. The standard deviation values range from 6.615 to 31.161, ultimately reducing the reliability of the findings. The % cytotoxicity for group H also presents a negative value at -18.382, which is significantly different from all other groups except B. The main factor influencing these results must have been a power-cut during the leaching period of the katana specimens in the media for 72 hours. This potentially resulted in the termination of the leaching procedure and the eppendorf tubes being positioned upside-down. This power-cut reduced the time the specimens were leached, ultimately preventing the optimum number of elements which could be suspended in the media. Furthermore, this led to the loss of some media through the small openings in the eppendorf tube lid. This could also have resulted in the specimens, which were previously anchored by the tubes, to become separated from the media . These issues encountered during the testing of this material have led to the results presented in figure 3.1.2. The findings from this material are therefore disregarded and the procedure would have to be repeated to gather accurate results in the future.

One-way ANOVA was also used for the analysis of the Celtra glaze and shades. The findings demonstrate a positive correlation between the percentage cytotoxicity and the application of the surface characterisation materials. The highest % cytotoxicity is shown for Group H which consists of glaze and 2 layers of the darker shade. This is in accordance with the assumptions that there is a greater ionic release from darker shades due to a superior amount of pigmentations present. The standard deviations for this material range from 19.638 to 84.121. This large range highlights the reduced accuracy of the results. The findings from the statistical analysis also indicates there is no

significant difference between some groups, which was not what was expected. An example is between group A, with no surface characterisations and group E, which has 2 layers of shades, which reduces the confidence in the results. These results gathered may be due to alterations in the methodology of the protocol used for the LDH assay in the cytotoxicity testing. The lysis buffer which was added to the positive control group remained incubated for 10 minutes, rather than the stated 45 minutes. This may have altered the maximum level of LHD activity readings and therefore the correct % cytotoxicity could not be calculated. This has therefore resulted in the increased values for standard deviation, although a clear trend in the % cytotoxicity can be seen. Due to these potential factors influencing the results shown in figure 3.3.1, the experiment would have to be repeated due to imprecise findings.

The ceramic heat-pressing / firing furnace was calibrated prior to the manufacturing process. This procedure ensured temperatures were accurate and followed the manufacturer's guidelines. Research has indicated repeated use of furnaces can oscillate the temperatures and limit their efficiency, therefore manufacturers recommend calibration following 50 pressing cycles. Problems in furnace calibration and temperatures can lead to porosities and changes in the mechanical and physical properties of materials (Gonzaga et al, 2008). This was important for this experiment as the quality of the glaze and shades must remain consistent throughout, in order to gain accurate results.

A limited number of metallic equipment and tools were used for the duration of this experiment. Designing custom-made zirconia firing stands and scoops allowed the standardisation of the procedure. Regarding the scoops, standardisation of the quantities of the materials used was of high importance, as this had not been shown accurately in

previous studies (Al-Wahadni and Martin, 1998). The scoops zirconia scoops allowed a contamination-free method of measuring the glaze and shade quantities, without the transfer of metal-oxides which would usually be present in metallic measuring equipment. This would lead to more reliable results, as unwanted ions would not be leached into the media during the cytotoxicity testing. Release of these ions could impact the biocompatibility of the material being tested, although the level of changes this may cause in the results is unknown (Anusavice, 1992).

The use of metallic firing stands was tested prior to the study to establish any potential issues which would be encountered. The metal stands showed evidence of contamination on the surface of the glaze and shades, due to sticking of the alloy residue. The curved tips of the custom zirconia stands ensured minimal contact would be made with the specimens. The grinding and polishing of the stands following each firing provided a smooth surface. This allowed for easier removal of the specimens.

Properties of the sprue wax used for the wax specimens can have a major impact on the quality of the pressed ceramic. Additives in the wax including silicon, pectin and alginic acid can cause deviations in the surface roughness and the quality of the pressed ceramic. The use of ash-free wax was adopted to sprue the glass-ceramic wax specimens. This was due to the materials ability to leave an oxide and residue-free mould following the burning out process. The absence of the ash also ensures no ash is left in the ceramic shell following the heat-pressing procedure. (Tascioglu and Akar, 2006). This was essential in the experiment as this extraneous factor could have influenced the final cytotoxicity results.

An irregular surface was observed on the ceramic specimens following the sand-blasting process. To overcome this issue, metallographic grinding was conducted on every surface of the glass-ceramic specimens to enable an even surface for the

application of the shades and glazes. This also allowed a standardised procedure as surface textures and areas were almost uniform. A light flow of water during the grinding allowed lubrication and prevented the silicon carbide paper from being over-abrasive. The grinding was not conducted on the zirconia specimens due to the excessive hardness of the material, and the time implications present in this study. Also, the specimen size differences were negligible during this process, as it had little impact on the results of the study. This procedure was also conducted in a similar cytotoxicity study by Brackett et al. (2008), who standardised the surface topography of their glass-ceramic specimens.

Ultrasonic cleaning using distilled water was conducted on each ceramic specimen following the grinding and sandblasting procedures. This was to ensure all debris, contaminants and potential metallic ions from the surfaces was removed. 50ml of distilled water was used for every 10 samples, and this was changed to ensure the water did not contain any impurities. The light vibration provided by the ultrasonic machine for 5 minutes allowed all the surfaces to be thoroughly cleaned. This has also been conducted in similar research by Hulterstrom and Bergman, (1993), who allowed their left their ceramic specimens in an ultrasonic bath for 30 minutes before assessing various polishing systems.

Sandblasting of ceramics according to manufacturer's guidelines is a vital procedure before the surface characterisation can be applied. The aim of this is to lightly abrade the surface of the specimens and create micro-texture for better adhesion of glazing and shading materials (Albakry et al., 2004). This also allows the surface to be cleaned and prepared before the application of the surface treatments. Any potential contamination of debris and extraneous particles from the surface of the ceramic can be removed. A study by Zalkind et al (1986) found that sandblasting with alumni oxide prior

to the application of the glaze, provided greater fineness on the surface resulting in a natural looking glazed surface.

In all cases, the glaze and shades were applied using a brush and light vibration to produce an even layer on the ceramic. Consistency in achieving the same thickness was essential to reproduce accurate results for the experiment. The vibration eliminated excessive air bubbles on the surface and prevented porosities following the firing of the ceramics A similar procedure was conducted in a study by Cattell et al, (2009), who used vibration to produce an even layer of dental glaze. Furthermore, the application of the external characterisation was conducted by a single person to ensure consistency in the procedure. A factor not that was not considered to influence the results was the volume of shade and glaze which was left over in the scoops or on the brush during the application procedure. This factor was therefore negligible and not worth consideration.

The use of serum-free media was adopted for the growth of the human dermal and gingival fibroblasts during the cytotoxicity testing, rather than serum-contain media. Serum containing media consists of hormones, haemoglobin and antioxidants to allow cell growth, however, the concentrations of these may vary. This form of media also enables the ability of designing the serum to be compatible to the cell line which is used, ultimately allowing regulation and differentiation (ATCC, 2012). Research by Moreira et al (1995) used baby hamster kidneys cells to compare their growth when in serum-free and serum-containing media. The results suggested cell viability and proliferation were very similar in both, with little variance during the final analysis. Therefore, this does not alter the validity of the results as any media type would be appropriate for use.

The addition of antibiotics in cell growth media is not necessary for the success of the experiment, however Penicillin Streptomycin (P/S) was added in the media as a safeguard to prevent potential microbial contamination. The use of the antibiotics is reliant

on many factors including its mechanism within the media and the cell-line being grown (Yang and Xiong, 2012). Biological infestation can be a result of poor laboratory practice, non-sterile pipette tips or insufficient incubation procedures. Contamination to the cells can result in changes of pH resulting in the cells becoming turbid. Microscopic observations are essential throughout to ensure the medium is suitable use prior to the investigations (Sigma-Aldrich, 2019).

As stated in the LDH assay protocol, the time duration for which the reaction mixture must be left in the 96-well plate must be 30 minutes before the addition of the stop solution. To ensue this was achieved, a time of 25 seconds was allowed between the addition of reaction mixture into 6 wells at a time. This provided consistency in time when the stop solution was inserted, increasing the validity of the research.

The 96-well plates were centrifuged prior to the light absorbance reading, as recommended by the manufacturer. This was done to remove any potential air-bubbles which could alter the light absorbance reading. A duration of 6 seconds for centrifuging allowed a long-enough time to remove the air without impinging on the readings themselves and causing separation of the liquid components (Lesk, 2016).

There were time limitations present in this study which reduced the amount of procedures that could be conducted for the cytotoxicity testing. An example of this was the inability to metallographically grind the zirconia specimens due to the hardness of the material. This was also due to the limited time available to conduct this study.

We were also unable to conduct PCR on all the materials due to a limited number of consumables available for this study, and due to time implications once again. Although this could have presented a better understanding of the impact of surface characterisations on gene expressions. For future research, PCR could be conducted if more time, equipment and consumables are available for the research.

5.0 Conclusion

Due to the problems encountered during the methodology of this experiment, 2 out of the 3 possible materials present unreliable results. Therefore, based on the e.max group alone, this study rejects the null hypothesis. This is due to the significance level for the cytotoxicity being at 0.005, which was lower than the 0.05 threshold set by us. We can therefore assume a relationship between the cytotoxicity and the application of dental glaze and shades on dental all-ceramic materials. The results for Katana and Celtra were not taken into consideration. The findings of this study provide an initiative to carry out further research into this topic area, to generate greater knowledge of the impact of cytotoxicity caused by dental glaze and shade.

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7.0 Appendices

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Light Absorbance is the same across categories of Group number and Surface treatment.	Independent-Samples Kruskal-Wallis Test	.005	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.